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Abstract: Clin Microbiol Infect 2010; 16: 1414-1420 ABSTRACT: Nasal carriage of Staphylococcus aureus contributes to an increased risk of developing an infection with the same bacterial strain. Genetic regulatory elements and toxin-expressing genes are virulence factors associated with the pathogenic potential of S. aureus. We undertook an extensive molecular characterization of methicillin-susceptible S. aureus (MSSA) carried by children. MSSA were recovered from the nostrils of children. The presence of Panton-Valentine leukocidin (PVL), exfoliatins A and B (exfoA and exfoB), and the toxic-shock staphylococcal toxin (TSST-1) and agr group typing were determined by quantitative PCR. A multiple-locus variable-number of tandem repeat analysis (MLVA) assay was also performed for genotyping. Five hundred and seventy-two strains of MSSA were analysed. Overall, 30% were positive for toxin-expressing genes: 29% contained one toxin and 1.6% two toxins. The most commonly detected toxin gene was tst, which was present in 145 (25%) strains. The TSST-1 gene was significantly associated with the agr group 3 (OR 56.8, 95% CI 32.0-100.8). MLVA analysis revealed a large diversity of genetic content and no clonal relationship was demonstrated among the analysed MSSA strains. Multilocus sequence typing confirmed this observation of diversity and identified ST45 as a frequent colonizer. This broad diversity in MSSA carriage strains suggests a limited selection pressure in our geographical area.

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Molecular epidemiology of the nasal colonization by methicillin-susceptible *Staphylococcus aureus* in Swiss children

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Abstract

Nasal carriage of *Staphylococcus aureus* contributes to an increased risk of developing an infection with the same bacterial strain. Genetic regulatory elements and toxin-expressing genes are virulence factors associated with the pathogenic potential of *S. aureus*. We undertook an extensive molecular characterization of methicillin-susceptible *S. aureus* (MSSA) carried by children. MSSA were recovered from the nostrils of children. The presence of Panton-Valentine leukocidin (PVL), exfoliatins A and B (exfoA and exfoB), and the toxic-shock staphylococcal toxin (TSST-1) and *agr* group typing were determined by quantitative PCR. A multiple-locus variable-number of tandem repeat analysis (MLVA) assay was also performed for genotyping. Five hundred and seventy-two strains of MSSA were analysed. Overall, 30% were positive for toxin-expressing genes: 29% contained one toxin and 1.6% two toxins. The most commonly detected toxin gene was *tst*, which was present in 145 (25%) strains. The TSST-1 gene was significantly associated with the *agr* group 3 (OR 56.8, 95% CI 32.0–100.8). MLVA analysis revealed a large diversity of genetic content and no clonal relationship was demonstrated among the analysed MSSA strains. Multilocus sequence typing confirmed this observation of diversity and identified ST45 as a frequent colonizer. This broad diversity in MSSA carriage strains suggests a limited selection pressure in our geographical area.

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Introduction

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. Three basic clinical syndromes are reported with this bacterium: (i) superficial lesions, such as wound infections and skin abscesses; (ii) systemic and deep-seated infections, such as bacteraemia, endocarditis, pneumonia and osteomyelitis; and (iii) toxaemic syndromes, such as staphylococcal scarlet fever, toxic shock syndrome (TSS), staphylococcal scalded-skin syndrome (SSSS), and staphylococcal food poisoning. Most *S. aureus* infections occur in individuals who are already colonized; accordingly, carriage has long been known to be one of the most strongly associated risk factors for developing subsequent infections, including bacteraemia [1,2]. A causal relationship between carriage and infection is further supported by the fact that the nasal carriage strain and the infecting strain very often possess similar genotypes. The prevalence of colonization in the paediatric population is in the range 18% to 52% [3]; younger children are significantly more often colonized than adolescents, and males more so than females. The ecological niche of *S. aureus* is mainly the anterior nares, although the skin, perineum and pharynx can also harbour the organism [4].

The expression of some *S. aureus* genes has been associated with defined diseases or syndromes. For example, the toxaemic syndromes are associated with the expression of genes encoding for specific exotoxins such as toxic-shock syndrome staphylococcal toxin (TSST-1) or exfoliatins, or for staphylococcal enterotoxins. Other genetic determinants of virulence coordinate the kinetics of the infection, such as the global accessory gene regulator *agr* [5], as recently reviewed [6]. *agr* comprises a regulatory element that controls the temporal expression of most virulence factors of *S. aureus* by simultaneously down-regulating surface proteins and up-regulating proteases when reaching the stationary phase. Strains can be further divided into four groups based on the sequence of this global regulator. Although this can be useful for typing purposes [7], these differences also explain bacterial interference, as observed when two strains from different *agr* types compete for the same niche [8]. In addition, associations were revealed between *agr*-III and *agr*-IV groups with TSS and SSSS syndromes, respectively [9,10]. A possible association between *agr* group and the acquisition of lowered susceptibility to glycopeptides was also recently suggested [11].

The prevalence of *S. aureus* nasal colonization has been studied across age categories and is highest in infants and young children, followed by a constant decline with age [12]. In the paediatric population, prevalence has been documented in specific countries but, to date, no systematic description of the molecular epidemiology has ever been performed on carriage isolates. In the present study, we performed extensive molecular characterization of MSSA carriage isolates, benefiting from a prospective multicentre Swiss study in children [13]. The analysis revealed an unsuspected genome-content diversity among the *S. aureus* strains not only across eight participating Swiss children's hospitals, but also within each studied centre.

Materials and Methods

Participants

Methodological details of this multicentre, cross-sectional study of children admitted to large Swiss teaching hospitals during a 1-month surveillance period (16 March 2006 to 15 April 2006) have been published elsewhere [14]. Briefly, the study took place in eight children's hospitals distributed throughout Switzerland, in Geneva, Lausanne, Bern, Basel, Zürich, Luzern, Aarau and St-Gallen. The first five institutions comprise all the tertiary university hospitals in Switzerland. Children 4 weeks of age or older admitted for inpatient care were eligible for enrolment. Exclusion criteria included in-house transfers of patients and newborns admitted to neonatal care directly after birth. After informed consent had been obtained from parents or guardians, a nasal swab from both nostrils was collected for culture of *S. aureus* within 48 h of admission, and comprehensive patient characteristics were entered into a standardized questionnaire.

Laboratory methods

One cotton swab per patient was used for both nostrils, placed in transport medium M40 Transystem™ (Copan, Brescia, Italy) and sent to the central bacteriological laboratory in Basel by overnight mail daily, except at weekends. For culture of *S. aureus*, a chromogenic medium (MRSA ID™ agar; bioMérieux, Marcy l'Etoile, France) and a selective enrichment broth (brain heart infusion broth with 6% NaCl; Biomedics, Madrid, Spain) were inoculated. Oxacillin resistance was tested by the use of a cefoxitin disk, according to the guidelines of Clinical and Laboratory Standards Institute (CLSA, formerly NCCLS) and as described previously [14]. After incubation overnight, the broth was subcultured on to another chromogenic agar for *S. aureus* (Chromagar Staph. aureus™; Hy Laboratories, Rehovot, Israel). Identification of *S. aureus* was based on various traits such as typical growth on a chromogenic medium and detection of clumping factor, protein A, and capsular antigens (PASTOREX STAPH-PLUS™; Bio-Rad, Marnes-la-Coquette, France). Isolates were then shipped to the Clinical Microbiology Laboratory of Geneva using a dedicated transport system, cultured on sheep blood agar and speciation was confirmed by PCR determination of the *femA* gene of *S. aureus* [15].

DNA extraction

Isolates were stored at −80°C in skim milk, directly from fresh plate cultures. Before experiments, isolates were thawed, plated on LB agar and assessed for purity. Genomic DNA was extracted from one colony suspended in 200 μL Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) in the presence of 100 mg of glass beads, as previously described [16].

Genotyping

Toxinotyping for the presence of Panton-Valentine leukocidin (PVL), exfoliatins A and B (exfoA and exfoB) and TSST-1 was performed by quantitative (q)PCR as previously described [17]. *agr* group typing was performed by a multiplex qPCR assay [7]. Genotyping, using a high-throughput multiple-locus variable-number of tandem repeat analysis (MLVA) assay, which consisted of a multiplex PCR with 10 primer pairs, was performed as previously reported [16,18]. In addition, a commercial repPCR assay [19] (DiversiLab; bioMérieux) was performed on the same strains, and multilocus sequence typ-

ing (MLST) according to the methods described by Enright *et al.* [20] on a representative panel of isolates.

Results

Study population

In total, 1736 children were eligible during this 1-month study period, representing 1762 hospitalizations. Of these, 356 patients (20.2%) were not included because of parental refusal (n = 88), > 48 h having elapsed subsquent to admission (n = 72) and various other reasons (n = 196), such as a short hospital stay with discharge before enrolment, language barriers, or the absence of parents. Finally, nasal swabs were available from 1363 hospitalizations in 1350 patients. Overall, 572 (42%) strains of MSSA and one strain of MRSA were isolated from 562 swabs. The fact that we considered 572 strains and not 562 as previously described [14] is explained by the better sensitivity of our genotyping analyses, allowing us to distinguish strains that were apparently similar and belonging to the same colony but that differed according to their MLVA profile, toxin content or *agr* group. The prevalence of nasal colonization with *S. aureus* was in the range 36% to 50% among the study sites (Table 1). Of 13 patients hospitalized twice, only four (31%) were carrying *S. aureus* at both investigation points. Ninety positive swabs (16%) were from children < 1 year old, 200 (36%) from children aged 1–6 years old, and 272 (48%) from children ≥7 years or more. Carriage occurred in 290 females (52%) and 272 males.

TABLE 1. Strain classification depending on *agr* group, toxin content and study site

agr	Toxin content	GE	VD	BE	ZH	AR	LU	SG	BS
Swabs		81	81	55	74	47	72	75	87
% MSSA		40.5	40.7	35.9	37.2	42.0	48.0	50.0	43.5
agr 1	TSST−, PVL−	23	28	30	24	24	32	30	44
	TSST+, PVL−	4	3	2	4	1	2	1	3
	TSST−, PVL+	0	0	0	0	0	0	0	1
agr 2	TSST−, PVL−	11	15	10	13	5	9	7	9
	TSST+, PVL−	2	0	0	1	0	0	1	0
	TSST−, PVL+	0	1	0	1	0	0	0	0
	TSST−, ExfoA+	1	3	0	0	0	0	0	0
agr 3	TSST−, PVL−	9	4	0	3	3	6	6	4
	TSST+, PVL−	20	21	10	18	4	17	15	12
	TSST−, PVL+	0	1	1	0	0	0	0	1
	TSST+, PVL+	0	0	0	0	0	0	0	0
	TSST−, ExfoA+	0	0	0	0	0	0	1	0
agr 4	TSST−, PVL−	7	2	2	7	8	5	7	8
	TSST+, PVL−	0	0	0	0	1	0	0	0
	TSST−, PVL+	0	1	0	0	0	0	0	1
	ExfoA+	1	0	0	1	0	1	4	2
	ExfoA+, PVL+	0	0	0	0	0	0	1	0
	ExfoA+, ExfoB+	1	1	0	2	0	0	2	1
agr ND	TSST−, PVL−	1	1	0	0	0	0	0	1
	TSST+, PVL−	1	0	0	0	1	0	0	0

GE, Geneva; VD, Lausanne; BE, Bern; BL, Basel; ZH, Zürich; LU, Luzern; AA, Aarau; SG, St-Gallen.

Three hundred forty-one children (61%) were of Swiss origin, 132 (24%) originated in Western Europe (Germany, England, France, Italy, Spain, Portugal, Belgium), 41 (8.4%) came from Eastern Europe (Kosovo, Turkey, Romania, Greece, Macedonia), 41 (7.3%) from other continents; and one (0.2%) had missing data.

Classification of *agr* groups

From 572 strains of *S. aureus*, 256 (45%) displayed *agr I*, 89 (16%) displayed *agr II*, 156 (27%) displayed *agr III*, 66 (12%) displayed *agr IV* type, and five (0.9%) remained unclassified. The different *agr* groups were distributed very similarly across the eight study centres (Fig. 1). However, in most centres, *agr* groups I and III were more frequently recovered and accounted for 66–79% of all strains (Table 1).

Distribution of toxin genes

A total of 172 strains (30%) of *S. aureus* were positive for toxin-expressing genes. Twenty-nine percent contained one toxin and 1.6% two toxins. The TSST-1 gene was the most common, present in 145 (25%) strains. Exfoliatins A, B, and PVL genes were less common, being recovered in 22 (3.8%), seven (1.2%) and nine (1.6%) strains, respectively.

Correlation between *agr* groups, toxin contents and demographic variables

We then attempted to correlate *agr* groups and toxin contents with the variables: hospital location; patient gender, age, and country of origin; previous antibiotic use during ≥7 days, recent hospital stay or hospitalization of a household member within the last 3 months. None of these variables could be linked to a particular *agr* group. However, a strong association was detected between the presence of the TSST-1 gene and *agr* group 3 (OR 56.8; 95% CI 32.0–100.8).

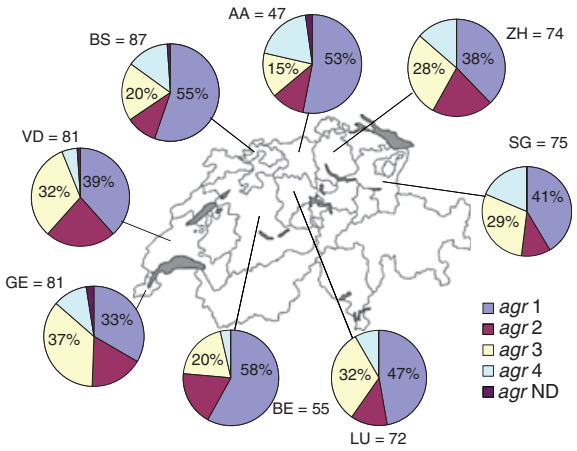

FIG. 1. *agr* repartition in study sites.

TABLE 2. Toxin expressing gene in the *agr* groups

agr	PVL+	PVL−	TSST− 1+	TSST− 1−	ExfoA+	ExfoA−	ExfoB+	ExfoB−
1 (*n* = 256)	1	255	20	236	0	256	0	256
2 (*n* = 89)	2	87	4	85	4	85	0	89
3 (*n* = 156)	3	153	118*	38	1	155	0	156
4 (*n* = 66)	3	63	1	65	17	49	7	59

**agr* 3/TSST-1 positive: significant association (OR 56.8, 95% CI 32.0–100.8).

The TSST-1 gene was present in only 8% of *agr* I group, 4% of *agr* II, 1.5% of *agr* IV, but 76% of *agr* III group (Table 2). Interestingly, analysis suggested that *agr* I was frequently associated with the absence of TSST-1 (237 of 512 strains).

No further significant association was demonstrated between any of the other three toxins (PVL, exfoA and exfoB) and the different *agr* groups.

Genotyping analysis

We performed a genotyping analysis to assess whether common clones were circulating in Switzerland. To allow for large numbers, we decided to concentrate our MLVA analysis on *agr* 3/TSST-1 positive strains recovered in all study sites. A RepPCR tree yielded to two major clusters of similar size. A homogeneous cluster composed of isolates showing sequence

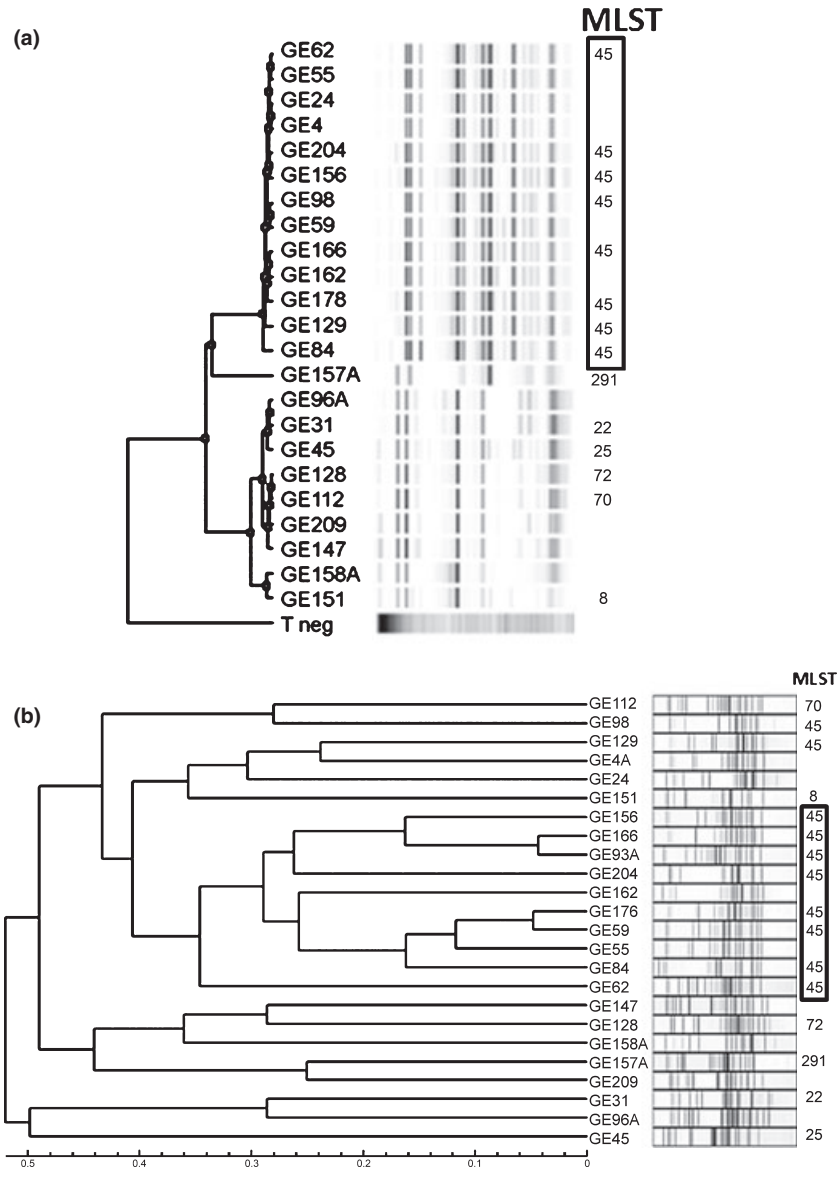

FIG. 2. Genotyping of *tst* positive isolates identified in Geneva. Clustering trees obtained using a commercially available repPCR assay DiversiLab (bioMérieux) (a) or with a multiple-locus variable-number of tandem repeat analysis (MLVA) assay (b). Sequence type of representative isolates are indicated to the right of each panel. Black rectangles indicate the position of the main cluster of isolates composed of ST45 strains. MLST, multilocus sequence typing.

type 45 (ST45) on the one hand, and a very heterogeneous cluster composed of strains appearing highly related on the other. Additionally, two isolates appeared to be totally unrelated to any other strain (Fig. 2a). MLST analysis confirmed that the main cluster was composed of clonal isolates with sequence type 45. The other cluster contained numerous different sequence types (Fig. 2a). Fig. 2b shows the tree obtained by a MLVA assay that allows various genetic backgrounds among the isolates to be distinguished. Only two pairs of isolates appeared genetically-related (GE176, 59 and 166, 93A) but the tree structure revealed an overall striking diversity of genetic content, with approximately 15 different combinations of repeated regions in this collection of strains and a large cluster composed of isolates identified as ST45 by MLST. The presence of the *tst* gene does not appear to be associated with any specific genetic background but rather to be dispersed across different unrelated strains.

Discussion

We analysed strains collected from 1350 children hospitalized in eight Swiss paediatric hospitals during a 1-month surveillance period. Overall, we identified 572 strains of *S. aureus* from 562 children, corresponding to an overall prevalence of 42%. Among the eight centres, carriage prevalence was in the range 36% to 50%, in the Bern and St-Gallen areas, respectively. This prevalence appears similar to that reported in other studies [21,22] but slightly higher than that observed in 2003 by Shopsin and Kreiswirth [23] who reported a 35% MSSA carriage in healthy children visiting a clinic. Only one child in our investigation was colonized with MRSA, and this parallels other studies that have shown that, despite an increase in MRSA prevalence in the hospital settings, MRSA carriage remains stable and low in children in the community [23,24], regardless of whether in the healthy population or in patients requiring hospitalization [25]. Overall, age, sex, use of antibiotics for ≥7 days and recent hospitalization of a household member were not found to be independent risk factors for TSST-1 positive *S. aureus* carriage or for a specific *agr*- group in our studied population. However, premature babies, and patients with acquired or congenital immunodeficiencies, with HIV, with intravascular devices, and those undergoing surgery are at increased risk of developing staphylococcal infections [26]. Efficient elimination of nasal colonization of strains carrying a specific genetic background could theoretically reduce the infection rates in specific populations identified as high risk groups [27,28]. Recently, Melles *et al.* [21] compared the strains recovered from HIV patients (who are prone to develop *S. aureus* infections with persistent carriage) with those recovered from healthy subjects and found them to be 'strongly overlapping' in genetic content assessed by the amplified fragment length polymorphism assay.

The accessory gene regulator (*agr*) is a crucial component of *S. aureus* involved in the temporal expression of numerous bacterial virulence factors. In our population, *agr* type I was the most abundant allele in seven of the eight geographical areas studied. Geneva was the only region with a majority of strains harbouring a type III *agr* allele. In the present study, the distribution of *agr* type was similar to that observed elsewhere [31], although no evidence of a massively predominant allele was found. Overall, our strain population contained 30% of isolates harbouring at least one potent toxin (e.g. exfoA or exfoB, TSST-1 or PVL). Most of the isolates with either of the two exfoliatin toxins belong to *agr* II and IV groups, which is consistent with the study of Jarraud *et al.* [30]. The TSST gene was predominantly found in strains harbouring *agr*-III, which also confirms a previous observation [10,29]. Our group recently published the epidemiological features of community-acquired MRSA in Geneva. In that study, we showed that 60% of community-associated strains harboured at least one toxin gene [17], which is twice the prevalence of strains found positive for similar toxins in this MSSA population. PVL was found in 1.6% of this MSSA cohort, which is a number significantly lower than that reported by others in infecting MSSA strains in southern European countries but strictly identical to the prevalence found in bacteraemia isolates in other European countries [32].

A broad genetic diversity was observed in this collection of *S. aureus* strains, and no clonal groups were observed in any of the centres. This suggests that the environmental selection pressure (e.g. of antimicrobial usage, country of origin, etc.) acting on *S. aureus* is very low in this population. A recent review suggested that colonizing and infecting strains share some common evolutionary features [33] partly related to environmental and host factors. The combination host-strain appears determinant in the co-evolution to either long-term asymptomatic carriage or to the development of staphylococcal infection [33].

Rapid genotyping using MLVA has been shown previously to provide at least similar discriminatory power to PFGE [16] and to allow further subdivision of groups of isolates that appear clonal when other genotyping methods such as MLST [17] or PFGE [16] are used. On our collection of strains harbouring the *tst* gene, MLVA was more discriminative than repPCR or MLST. MLST and repPCR identified a major cluster of ST45 isolates and numerous individual isolates of various phylogenetic origins. The MLVA assay shows the striking diversity of genetic backgrounds in our

population, even among ST45 isolates. This observation is in accordance with a previous report on MRSA showing that ST45 encompasses a rather broad spectrum of strains with different genetic features and staphylococcal cassette chromosome *mec* (*SCCmec*) elements [34]. The properties of such typing strategies were previously reported by Tenover *et al.* [19] who showed that DiversiLab was significantly less discriminative than PFGE. A previous report on MSSA from German carriers showed that the *tst* gene was mainly harboured by strains from the CC30 group [35], a finding that differs from the paediatric carriage as observed in our geographical area. This was confirmed by the absence of clusters containing strains harbouring similar *agr* type or toxin content. This observation is strikingly different from a similar analysis performed on MRSA populations, which suggested that a limited number of genetic contents only are able to receive a SCC*mec* element [17]. Surprisingly, this extreme diversity of genetic backgrounds has also been observed very recently in children colonized by methicillin susceptible *Staphylococcus epidermidis* [36]. This suggests that the restriction observed in MRSA is rather a result of antibiotic pressure than to the genetic background.

Our analysis has some limitations. The cross-sectional design only measures carriage prevalence and no serial cultures were achieved. The children enrolled in the study had been admitted to hospital and are therefore not necessarily representative of the whole children's population in the country. Despite the fact that the present study was designed to determine the carriage rates of MSSA and MRSA, most clinical and epidemiological items in the questionnaire were based on the literature regarding MRSA.

Our observations suggest that the environmental selection pressure on *S. aureus* is likely to be moderate in this population. Further work would allow us to discover whether infectious strains share the same genotypic characteristics as colonizing strains, within the same patient population.

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Transparency Declaration

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